

Detection of Residual Neuroblastoma Cells in Bone Marrow: Comparison of Flow Cytometry With Immunocytochemistry

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Background: Because the cytomorphologic examination of bone marrow (BM) aspirates appears not sensitive enough to detect residual neuroblastoma cells, two four-color flow cytometric assays using different combinations of CD9, CD81, CD56, CD45, and anti-GD2 were evaluated.

Methods: The sensitivity of the flow cytometric assays was assessed by spiking experiments in normal peripheral blood samples. Twenty-eight BM samples, 12 biopsies, and 3 peripheral blood stem cell (PBSC) preparations from 22 patients with neuroblastoma were analyzed. The results were compared with those of an anti-GD2 immunocytochemical reference assay.

Results: Flow cytometric and immunocytochemical analyses showed residual neuroblastoma cells in four BM samples. One PBSC preparation and 20 BM samples were negative for both assays. Four BM and two PBSC samples scored positive for the immunocytochemical assay but were negative for the flow cytometric tests. This was due to the limited number of cells that were flow cytometrically analyzed. A strong correlation between the flow cytometric and immunocytochemical tests was found ($\chi^2 = 6.4$, $P = 0.011$).

Conclusions: When an equal amount of cells is analyzed, the sensitivity of the flow cytometric assays is to be about 10 times lower than that of the immunocytochemical test. However, the flow cytometric assays can be used to screen for residual cells in clinical samples with a sensitivity of one neuroblastoma cell in 10^4 to 10^5 normal mononuclear cells. Flow cytometry is simple, quick, and cost effective compared with immunocytochemistry. In addition, the flow cytometric assays can be used to screen for residual neuroblastoma cells in case of a GD2-negative primary tumor. Therefore we recommend flow cytometry for the detection of residual neuroblastoma cells. © 2004 Wiley-Liss, Inc.

Key terms: neuroblastoma; minimal residual disease; flow cytometry; immunocytochemistry

Neuroblastoma (NB), a tumor originating from the sympathetic nervous tissue, is the most common extracranial solid tumor in children. It shows a wide range of biologic, genetic, and morphologic characteristics and exhibits a diverse clinical behavior. Some children present with a localized tumor associated with a good prognosis, whereas others have a highly metastatic, aggressive tumor with an unfavorable outcome (1).

Approximately 40% of patients with NB have high-risk stage 4 disease with bone marrow (BM) involvement. These children have a poor clinical outcome, with a 5-year survival rate of approximately 30% (2). The presence of NB cells in the BM during therapy can predict relapse or clinical outcome (3–6). The detection of BM involvement is thus critical for accurate staging and risk assessment.

Myeloablative chemotherapy in combination with autologous BM or peripheral blood stem cell (PBSC) transplantation is commonly used to treat children with stage 4 disease (7). Residual NB cells can contaminate the stem cell product (8). This can cause the recurrence of the disease due to the reinfusion of neuroblastoma cells (9).

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Therefore, stem cell preparations must be screened for the presence of residual neuroblastoma cells (10).

The cytomorphologic examination of BM aspirates is not sensitive enough to detect residual NB cells (11). More sensitive assays, based on the use of immunocytochemistry, reverse transcription-polymerase chain reaction (RT-PCR) or flow cytometry, were developed to screen for NB cells (12-19).

Komada et al. evaluated a triple-color flow cytometric assay using CD9 in combination with CD56 and CD45 (12). This test was optimized by Nagai et al. (14) who combined CD81, CD56, and CD45. Warzynski et al. tested several combinations (18). They used CD45, CD56, a monoclonal antibody against neuron-specific enolase (NSE) and anti-GD2. GD2 disialoganglioside is highly expressed in almost all NBs and the GD2 density in NB cells is high (5 to 10×10^6 molecules/cell) (20-23). Warzynski et al. evaluated different double- and triple-color assays and analyzed anti-GD2, CD81, CD45, and CD56 expressions of the NB cell line SK-N-DZ by using a four-color flow cytometric assay that they currently use to evaluate their clinical samples (18).

In this study, we developed a new four-color flow cytometric assay combining CD9, CD81, CD56, and CD45. In addition, we evaluated CD81, CD56, and CD45 in combination with an anti-GD2 monoclonal antibody.

CD9 reacts with a 24-kDa type III transmembrane protein that is expressed on platelets, pre-B cells, activated and differentiating B cells, activated T cells, eosinophils, basophils, monocytes, endothelial and epithelial cells, brain, peripheral nerves, and muscular cells. Various *in vitro* studies have shown consistent expression of the CD9 antigen on NB cells (24,25).

The expression of CD81 in neuroblastoma cell lines has also been described (26). CD81 identifies a 26-kDa type III transmembrane protein (TAPA-1) that is involved in cell growth and signal transduction. It is broadly expressed on cells of hematopoietic origin. In addition, it is present on endothelial and epithelial cells.

CD56 reacts with the 175-/220-kDa glycosylated antigen isoforms present on a subpopulation of peripheral blood, large, granular lymphocytes that demonstrate natural killer activity. It is also present on a subset of CD4⁺ and CD8⁺ T cells in peripheral blood, neural-derived cells and tumors, large granular lymphocyte and myeloid leukemias, small cell lung carcinomas, and myelomas. The expression of neural cell adhesion molecule in human neuroblastoma cells lines has been reported by Lipinski et al. (27).

GD2 disialoganglioside is expressed by neuroectodermally derived tumors such as melanoma, neuroblastoma, small cell carcinoma, and glioma. GD2 expression is not found in normal BM or peripheral blood samples (28).

CD45 reacts with the isoforms of the leukocyte common antigen present on all human white blood cells, including lymphocytes, monocytes, granulocytes, eosinophils, and thymocytes. It is not expressed in NB cell lines (29).

We analyzed 12 tumor samples from patients with NB and used the four-color flow cytometric assays to screen for residual NB cells in 28 BM samples and three PBSC

preparations. The sensitivity of the flow cytometric assays was evaluated with spiking experiments. We assessed the specificity of our four-color flow cytometric tests by analyzing samples from a ganglioneuroma, a Wilms tumor, a lung sequester, a rhabdomyosarcoma, and a desmoplastic infantile ganglioglioma (DIG). Wilms tumors and rhabdomyosarcomas, like NBs, Ewing sarcomas, non-Hodgkin lymphomas, retinoblastomas, medulloblastomas, some small cell carcinomas, and primitive neuroectodermal tumors, belong to the family of "small blue round cell" neoplasms. Because the members of this family can often be morphologically confused with NB, the two four-color flow cytometric assays could be used to differentiate between NB and the other neoplasms.

The results of the flow cytometric assays were compared with those of an anti-GD2 immunocytochemical test, which was considered the reference method.

MATERIALS AND METHODS

Patients and Sample Preparation

Between May 2001 and August 2003, 12 biopsies, 28 BM samples, and 3 PBSC preparations from 22 patients with NB, taken at diagnosis or during treatment, were examined. In addition, samples of a ganglioneuroma, a Wilms tumor, a lung sequester, a rhabdomyosarcoma, and a DIG were analyzed. Patient characteristics are summarized in Table 1. Bilateral BM samples were considered as two samples because they were analyzed separately.

Clinical staging was done according to the International Neuroblastoma Staging System (1). The ethical committee approved the study, and informed consent was obtained from patients and/or their parents.

From fresh tumor samples, single-cell suspensions were prepared by using collagenase (1,000 U/ml in RPMI medium) for 1 h at 37°C. Subsequently, cells were washed and resuspended, and cell clumps were removed by filtering through a 70- μ m nylon membrane.

Immediately after collection, mononuclear cells from BM samples and PBSC preparations were isolated by density gradient centrifugation on Ficoll-Hypaque.

All cells were kept at 4°C and analyzed within 24 h after collection.

Control Samples

The NB cell line CLB-GA (kindly provided by F. Speleman, Medical Genetics, Ghent University Hospital, Ghent, Belgium) was included in the experiments as a positive control. BM samples from 10 adults without malignant disease were used as negative controls and were analyzed with the flow cytometric and immunocytochemical tests.

Monoclonal Antibodies

NB cells were detected by a four-color flow cytometric assay using five different monoclonal antibodies (CD9, CD81, CD56, anti-GD2, and CD45). We evaluated CD9 (clone P1/33/2) labeled with fluorescein isothiocyanate (FITC), CD81 (clone JS-81) labeled with phycoerythrin (PE), CD45 (clone 2D1) labeled with peridinin chloro-

Table 1
Patient Characteristics^a

Patient	Diagnosis	Stage	Age (months)	Sex	Sample	Time point ^b
A	NB	4	87	M	Biopsy	Diagnosis
B	NB	3	13	F	Biopsy	Diagnosis
					BM left	Diagnosis
					BM right	Diagnosis
C	NB	1	27	M	Biopsy	Diagnosis
D	NB	4	93	M	Biopsy	Diagnosis
E	NB	4	22	M	Biopsy	Diagnosis
					Biopsy	16
					BM	28
F	NB	4S	9	F	Biopsy	Diagnosis
G	NB	3	160	F	Biopsy	Diagnosis
					BM left	Diagnosis
H	NB	4	267	M	Biopsy	Relapse
I	NB	2	36	F	Biopsy	Diagnosis
J	NB	1	29	M	Biopsy	Diagnosis
K	NB	1	32	F	Biopsy	Diagnosis
L	NB	4	49	F	BM	Diagnosis
M	NB	4S	31	F	BM left	Diagnosis
					BM right	Diagnosis
N	NB	4	74	M	BM left	Diagnosis
					BM	7
					BM right	13
					PBSC 1	13
					PBSC 2	13
O	NB	3	34	M	BM	Diagnosis
P	NB	3	21	F	BM	Diagnosis
Q	NB	4	53	M	BM	Diagnosis
R	NB	3	44	F	BM left	Relapse
					BM right	Relapse
S	NB	4S	43	F	BM	59
T	NB	4	62	M	BM	13
					PBSC	13
					BM left	23
					BM right	23
					BM right	40
U	NB	4	71	F	BM	Diagnosis
					BM	11
V	NB	4	106	F	BM left	Diagnosis
					BM right	Diagnosis
					BM left	7
					BM right	7
					BM left	13
					BM right	13
W	DIG	NA	25	F	Biopsy	11
X	GN	NA	179	F	Biopsy	Diagnosis
Y	Wilms	NA	55	F	Biopsy	Diagnosis
Z	LS	NA	17	M	Biopsy	Diagnosis
AA	RM	NA	103	M	BM	Diagnosis

^aNB, neuroblastoma; DIG, desmoplastic infantile ganglioglioma; GN, ganglioneuroma; Wilms, Wilms tumor; LS, lung sequester; RM, rhabdomyosarcoma; M, male; F, female; BM, bone marrow; NA, not applicable. PBSC, peripheral blood stem cell.

^bWeeks after diagnosis.

phyll protein (PerCP), CD56 (clone N901) labeled with allophycocyanin (APC), and an unlabeled monoclonal antibody that interacts with GD2 disialoganglioside (clone 14G2a).

CD9 was purchased from Dako Corporation (Glostrup, Denmark); CD81, CD45, and anti-GD2 were obtained from BD Biosystems (Erembodegem, Belgium); and CD56 was purchased from Beckman Coulter (Fullerton, CA, USA).

Flow Cytometric Assay

Mononuclear cells (5×10^5 cells) isolated from BM samples, PBSC preparations, or fresh tumor samples were resuspended in 100 μ l RPMI medium and incubated for 30 min with an unlabeled isotypic control antibody immunoglobulin G2a as a control for the GD2 assay (5 μ l; Dako Corporation). In parallel, cells were incubated with 5 μ l CD9-FITC, 5 μ l CD81-PE, 5 μ l CD45-PerCP, and 3 μ l

CD56-APC. In addition, 5×10^5 cells were incubated with 2 μ l of the unlabeled anti-GD2 antibody, which was diluted 1:50 in phosphate buffered saline (PBS). The volumes of the different antibodies were titrated against the CLB-GA cell line.

The cells were washed twice with PBS. The samples incubated with the isotypic control antibody or the anti-GD2 antibody were incubated for another 30 min with a goat F(ab')₂ anti-mouse immunoglobulin G antibody labeled with FITC (1 μ l; Caltag Laboratories, Burlingame, UK). After another wash with PBS, we incubated the cells, which had been incubated with the anti-GD2 antibody, with 5 μ l CD81-PE, 5 μ l CD45-PerCP, and 3 μ l CD56-APC. The cells were washed twice and resuspended in PBS. The fluorescence was analyzed on a FACSort flow cytometer (BD Biosystems). At least 10,000 events were counted and analyzed with Cell Quest software (BD Biosystems). NB cells were positive for GD2, CD9, CD81, and CD56 but negative for CD45. Therefore, percentages of CD9⁺/CD81⁺/CD45⁻/CD56⁺ and GD2⁺/CD81⁺/CD45⁻/CD56⁺ cells were calculated by using the following gating strategy. On a forward scatter/side scatter dot plot, the mononuclear cells were identified by region R1. In all other dot plots, only the cells lying in R1 were represented. Region R2 defined the CD56⁺/CD45⁻ cells. The anti-GD2⁺/CD81⁺ or CD9⁺/CD81⁺ cells were identified by region R3. Samples were considered positive when at least 10 to 20 cells clustering together in R1, R2, and R3 were detected (30).

Spiking Experiments

The sensitivity of the four-color flow cytometric and the immunocytochemical assays was evaluated by using spiking experiments. Cells from NB cell line CLB-GA were added to peripheral blood mononuclear cells that had been isolated from healthy volunteers. The concentrations of the NB cells in this dilution experiment were 1%, 0.1%, 0.01%, 0.001%, and 0.0001%. The cells were suspended in RPMI medium.

The dilutions from 1% to 0.001% were evaluated flow cytometrically. With the different dilutions, 3×10^4 , 5×10^4 , 3×10^5 , and 2×10^6 events were acquired.

The three highest dilutions (0.01% to 0.0001%) were immunocytochemically analyzed, and 5×10^5 , 1×10^6 , and 3×10^6 cells were evaluated. Because convincing results were obtained, the experiments were performed only once.

Immunocytochemistry

After air drying and fixation with 4% paraformaldehyde, large-diameter (17 mm) cytopins (Hettich Zentrifugen, Tuttlingen, Germany) containing 5×10^5 mononuclear cells were incubated with 30 μ l of an unlabeled monoclonal antibody against GD2 disialoganglioside (clone 14G2a, diluted 1:100 in PBS) in a humidifier at room temperature for 30 min. Controls, in which the primary antibody was replaced by PBS buffer, were included to evaluate the background staining. During a second and a third incubation step, cells were incubated for 30 min with 30 μ l of a

polyclonal goat anti-mouse antibody (diluted 1:20 in PBS; Dako Corporation) and 30 μ l of a polyclonal rabbit anti-goat antibody (diluted 1:20 in PBS; Dako Corporation), respectively. Both antibodies were labeled with alkaline phosphatase (31). The bound alkaline phosphatase complexes were stained with the new fuchsin chromogen (Sigma, St. Louis, MO, USA) (32). The slides were counterstained with hematoxylin (Sigma) and examined under a light microscope by two independent observers. Preferably six slides, each containing 5×10^5 cells, were analyzed. Cells with a round nucleus, a granular chromatin structure, a sparse amount of cytoplasm, and a strong staining around the entire cell were considered positive. Cells with an aberrant morphology or a pink staining restricted to a subcellular compartment were considered negative. The number of positive single cells and positive cells being part of a Homer-Wright rosette or cell clump were counted. The number of evaluated mononuclear cells was also reported. When the two local observers disagreed or fewer than 10 positive cells were found, pictures of the doubtful cells were sent to an external expert. The results were discussed until agreement was reached.

Statistics

After classifying the results in a frequency table, a χ^2 test was performed to study the relation between the different categories. In addition, a κ test was used to measure the agreement between the different assays.

RESULTS

Phenotype of NB Cells

Cells from the NB cell line CLB-GA were phenotyped with the four-color flow cytometric assay. The CLB-GA cells showed a strong expressions for GD2, CD9, CD81, and CD56 and were negative for CD45 (Fig. 1).

The same markers were analyzed in NB cells from 12 tumor samples. All samples were positive for CD9, CD81, and CD56 and negative for CD45. In one sample, the NB cells lacked GD2 expression. This result was confirmed by immunocytochemistry.

Ten BM samples from healthy donors were included as negative controls. None of these samples scored positive on the immunocytochemical or flow cytometric tests.

Specificity of the Four-Color Flow Cytometric Assay

We assessed the specificity of the four-color flow cytometric tests by analyzing tumor samples from a DIG, a ganglioneuroma, a Wilms tumor, a lung sequester, and a rhabdomyosarcoma.

The cells from the DIG were CD9⁺/CD81⁺/CD56⁺/CD45⁻ and GD2⁺/CD81⁺/CD56⁺/CD45⁻. Consequently, it was impossible to distinguish between a NB and a DIG by using our four-color flow cytometry assay. The immunocytochemical test was negative because the DIG cells did not meet the morphologic criteria.

Based on our limited results, it was possible to differentiate between NB and the other tumors by using the four-color flow cytometric assay. Tumor cells from the

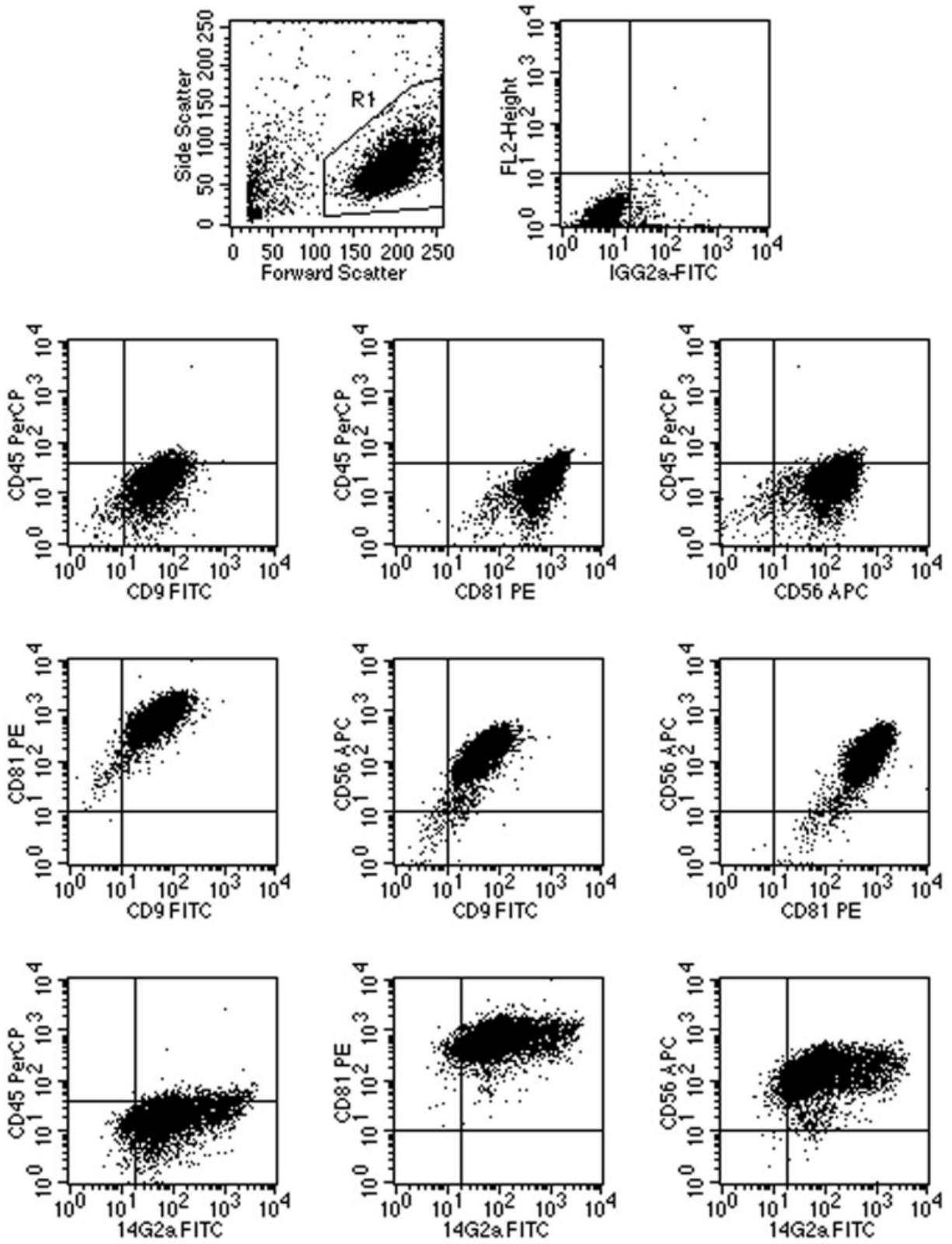


FIG. 1. Phenotype of the neuroblastoma cell line CLB-GA. The CLB-GA cells show strong GD2, CD9, CD81, and CD56 expressions and are negative for CD45 (immunoglobulin G2a [IGG2a], isotypic control; 14G2a, anti-GD2 monoclonal antibody).

Table 2
Results of BM and PBSC Preparations

Patients	Samples	Stage	Time point ^a	Flow Cytometry	Cells evaluated	Immunocytochemistry	Cells evaluated
L	BM 1	4	Diagnosis	—	2,00E + 04	—	1,00E + 06
B	BM 2	3	Diagnosis	—	2,00E + 04	—	5,00E + 05
	BM 3	3	Diagnosis	—	2,00E + 04	—	7,00E + 05
E	BM 4	4	28	—	2,00E + 04	—	1,70E + 06
M	BM 5	4S	Diagnosis	—	2,00E + 04	—	1,00E + 06
	BM 6	4S	Diagnosis	—	2,00E + 04	—	1,00E + 06
N	BM 7	4	Diagnosis	+	2,00E + 04	+	3,00E + 06
	BM 8	4	7	—	2,00E + 04	—	2,50E + 06
	BM 9	4	13	+	3,38E + 05	+	1,50E + 06
O	BM 10	3	Diagnosis	—	2,00E + 04	—	3,00E + 06
P	BM 11	3	Diagnosis	—	2,00E + 04	—	3,00E + 05
Q	BM 12	4	Diagnosis	—	2,00E + 04	—	1,50E + 06
G	BM 13	3	Diagnosis	—	2,00E + 04	—	5,00E + 05
R	BM 14	3	Relapse	—	2,00E + 04	—	1,50E + 06
	BM 15	3	Relapse	—	2,00E + 04	—	1,50E + 06
S	BM 16	4S	59	—	2,00E + 04	—	4,00E + 05
T	BM 17	4	13	—	2,00E + 04	+	1,00E + 06
	BM 18	4	23	—	2,00E + 04	—	7,00E + 05
	BM 19	4	23	—	2,00E + 04	—	1,20E + 06
	BM 20	4	40	—	2,00E + 04	+	9,00E + 05
U	BM 21	4	Diagnosis	—	2,00E + 04	—	7,00E + 05
	BM 22	4	11	—	2,00E + 04	—	4,00E + 05
V	BM 23	4	Diagnosis	+	2,00E + 04	+	1,40E + 05
	BM 24	4	Diagnosis	+	2,00E + 04	+	1,00E + 05
	BM 25	4	7	—	2,00E + 04	—	3,00E + 06
	BM 26	4	7	—	2,00E + 04	—	3,00E + 06
	BM 27	4	13	—	2,00E + 04	+	2,00E + 05
	BM 28	4	13	—	2,00E + 04	+	9,50E + 05
N	PBSC 1	4	13	—	1,00E + 05	+	3,00E + 06
	PBSC 2	4	13	—	4,00E + 05	—	2,50E + 06
T	PBSC 3	4	13	—	2,00E + 04	+	1,00E + 06

^aWeeks after diagnosis, BM, bone marrow; PBSC, peripheral blood stem cell.

ganglioneuroma and the lung sequester showed weak CD56 and GD2 expressions. The Wilms tumor was negative for CD56 and GD2 and showed only weak CD9 and CD81 expressions. Rhabdomyosarcoma cells were negative for GD2. None of these tumors scored positive for the immunocytochemical assay.

Sensitivity Experiments

The sensitivity of the flow cytometric assays was evaluated with spiking experiments. CLB-GA cells were diluted into normal mononuclear cells that had been isolated from peripheral blood taken from healthy volunteers. Four different dilutions (1%, 0.1%, 0.01%, and 0.001%) were analyzed. As few as one NB cell in 10^4 to 10^5 normal mononuclear cells could be detected with the four-color flow cytometric assays. Regression analysis was performed:

$$\text{CD9/CD81/CD45/CD56: } \log(Y) = 0.41 + 1.09 \log(X)$$

standard error of the slope = 0.10, corresponding

$$P = 0.0082, \text{ residual standard deviation} = 0.22$$

$$\text{GD2/CD81/CD45/CD56: } \log(Y) = 0.44 + 1.23 \log(X)$$

standard error of the slope = 0.13, corresponding

$$P = 0.010, \text{ residual standard deviation} = 0.28$$

Both tests performed equally well during the spiking experiment.

It is important to analyze a sufficient number of cells when the tumor load in a sample is low ($<0.01\%$) because samples can be considered positive only when at least 10 to 20 cells with the correct immunophenotype, clustering together in the different dot plots, are detected. In our study, at least 2×10^4 cells were flow cytometrically analyzed. This means that a sensitivity of 0.1% to 0.2% was reached.

The sensitivity of the immunocytochemical test was also evaluated with spiking experiments. Three different dilutions (0.01%, 0.001%, and 0.0001%) were analyzed. When 3×10^6 cells were evaluated, one NB cell in 10^6 normal mononuclear cells could be detected.

Detection of NB Cells in BM and PBSC Preparations

Twenty-eight BM samples and three PBSC preparations were analyzed with the flow cytometric assay. The results are summarized in Table 2. Flow cytometry scored positive in four BM samples, and 24 samples were negative.

Table 3
Discordant Flow Cytometric and Immunocytochemical Results

Patients	Samples	Stage	Time point ^a	Flow cytometry	Cells evaluated	Immunocytochemistry	Cells evaluated	No. of positive cells
T	BM 17	4	13	—	2,00E + 04	+	1,00E+06	5
	BM 20	4	40	—	2,00E + 04	+	9,00E+05	4
V	BM 27	4	13	—	2,00E + 04	+	2,20E+05	2
	BM 28	4	13	—	2,00E + 04	+	9,50E+05	3
N	PBSC 1	4	13	—	1,00E + 05	+	3,00E+06	2
T	PBSC 3	4	13	—	2,00E + 04	+	1,00E+06	5

^aWeeks after diagnosis, BM, bone marrow; PBSC, peripheral blood stem cell.

These results were compared with the results of an immunocytochemical assay. We found concordant results in 24 of 28 BM samples (86%). The four samples were positive for the flow cytometric assay and for the immunocytochemical test. Twenty BM samples were negative for both assays. During therapy, discordant results were found in four BM samples and two PBSC preparations. Although these samples scored positive for the immunocytochemical test, fewer than 10 cells clustering together in the different dot plots were found after four-color flow cytometric analysis. In these samples, the number of residual NB cells was very small (Table 3). In addition, there was a significant difference in the number of mononuclear cells analyzed by both techniques. Using flow cytometry, an average of 3.3×10^4 cells was analyzed. In contrast, an average of 1.2×10^6 cells was evaluated using immunocytochemistry.

Figure 2 shows an example of a BM sample that scored positive for the flow cytometric and immunocytochemical assays. Figure 3 shows an example of discordant results. Three positive cells were detected after immunocytochemical analysis of 3×10^6 mononuclear cells. Because only 5×10^5 cells were flow cytometrically analyzed, theoretically only 0.5 tumor cells could have been detected by the flow cytometric assays. Both samples were only recently analyzed and are not included in the statistically processed data.

There was no change in the intensity of the antigen expression or the immunophenotype of the tumor cells when, on more than one occasion, NB cells were detected.

Based on the results of the BM and PBSC samples, an association between the flow cytometric and immunocytochemical test was found ($\chi^2 = 6.4$, $P = 0.011$, $df = 1$). In addition, the κ statistic showed a moderate level of agreement between tests ($\kappa = 0.48$).

DISCUSSION

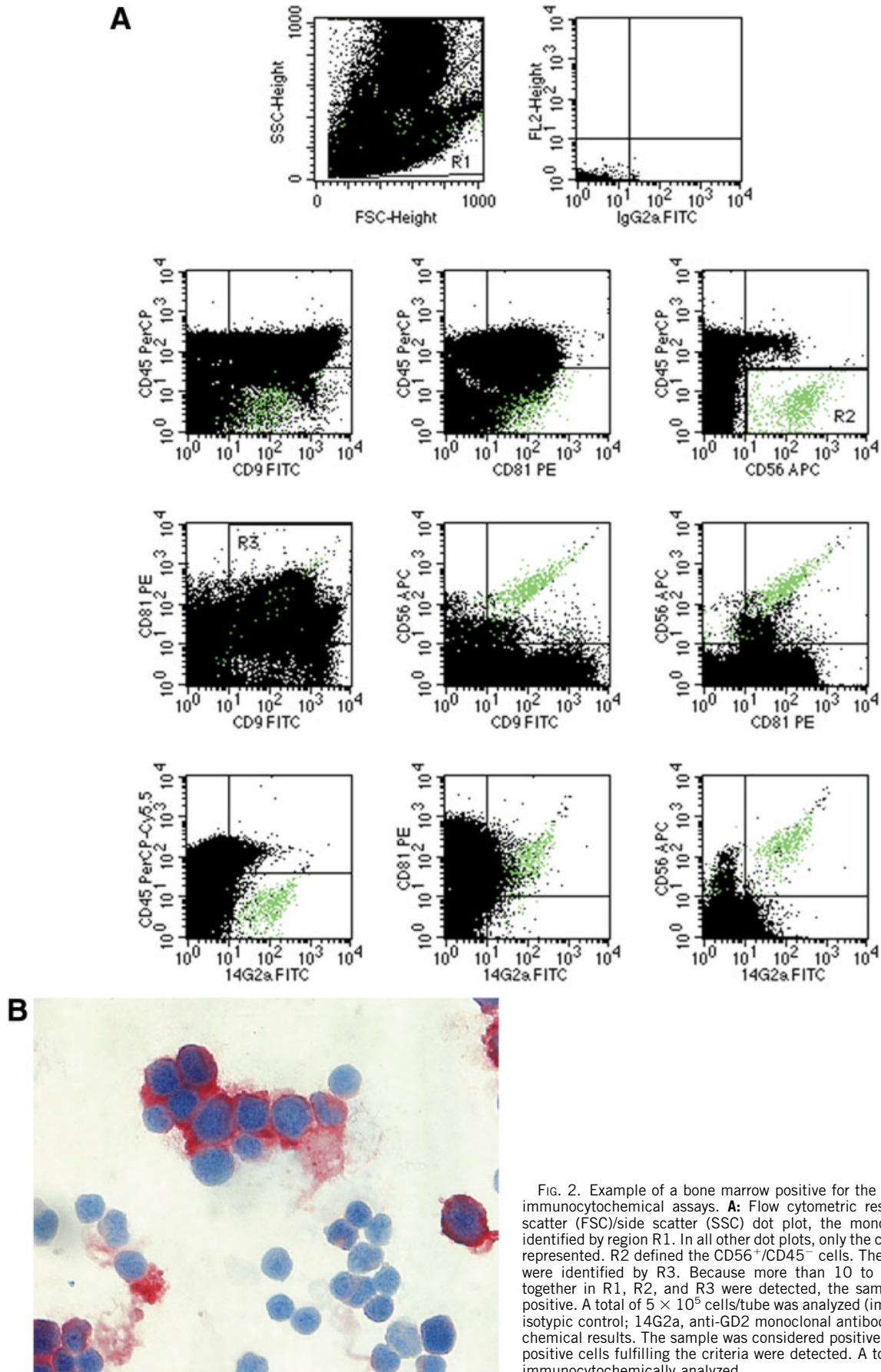
Many children with NB present with disseminated disease, including BM involvement. The detection of contaminating NB cells in the BM plays an important role in clinical staging and risk assessment. Moreover, the detection of disseminating tumor cells in the autologous stem cell preparations may signify the need for ex vivo manipulations such as tumor cell purging and/or CD34⁺ cell selection to avoid the reinfusion of malignant cells (33).

Different techniques for the detection of residual NB cells such as immunocytochemistry, RT-PCR, and flow cytometry are described in the literature (12–19).

Flow cytometry has been used in the clinical practice of hematology and oncology for many years and has many advantages compared with immunocytochemistry or RT-PCR. The flow cytometric assay provides information on the antigen expression from a single tumor cell in a multicolor assay and allows the analysis of large cell numbers. In addition, flow cytometry, like automated fluorescence microscopy, allows a more objective quantification of the number of NB cells compared with conventional immunocytochemistry or quantitative RT-PCR (17,34).

In this study, we developed a new four-color flow cytometric assay using CD9, CD81, CD45, and CD56 to detect NB cells in BM. A second four-color assay based on the use of anti-GD2, CD81, CD45, and CD56 was evaluated (18). Twelve NB tumor samples were analyzed. One tumor lacked GD2 expression. This result was confirmed by the immunocytochemical assay. The sample was taken from a 13-year-old girl with a palpable mass in the abdomen. The NSE serum concentration and the urinary dopamine concentration were elevated. Magnetic resonance imaging showed a large tumor extending from the right kidney to the pelvis and into the lumbar foramina. No metastases were found. Immunohistologically, the tumor cells showed strong CD56 and NSE expressions. They were moderately positive for chromogranin and synaptophysin. There were no obvious signs of cytodifferentiation. After genetic analysis, no MYCN amplification or 1p deletion was found, but 70% of the tumor cells had an extra copy of chromosome 17 and 18% showed 17q gain. The diagnosis of a stroma-poor, poorly differentiated, stage 3 NB was made for this very unusual GD2-negative case. Because nearly all NBs express high levels of GD2 (21–23), immunotherapy using anti-GD2 antibodies is considered a promising treatment strategy (35). However, when the primary tumor lacks GD2 expression, treatment with anti-GD2 immunotherapy is useless. Therefore, we recommend the evaluation of GD2 expression in tumor samples from all NB patients at diagnosis.

It can be difficult to differentiate between NB and other members of the small blue round cell tumor family. Our results suggest that the four-color flow cytometric assays are helpful in making a differential diagnosis. Only cells



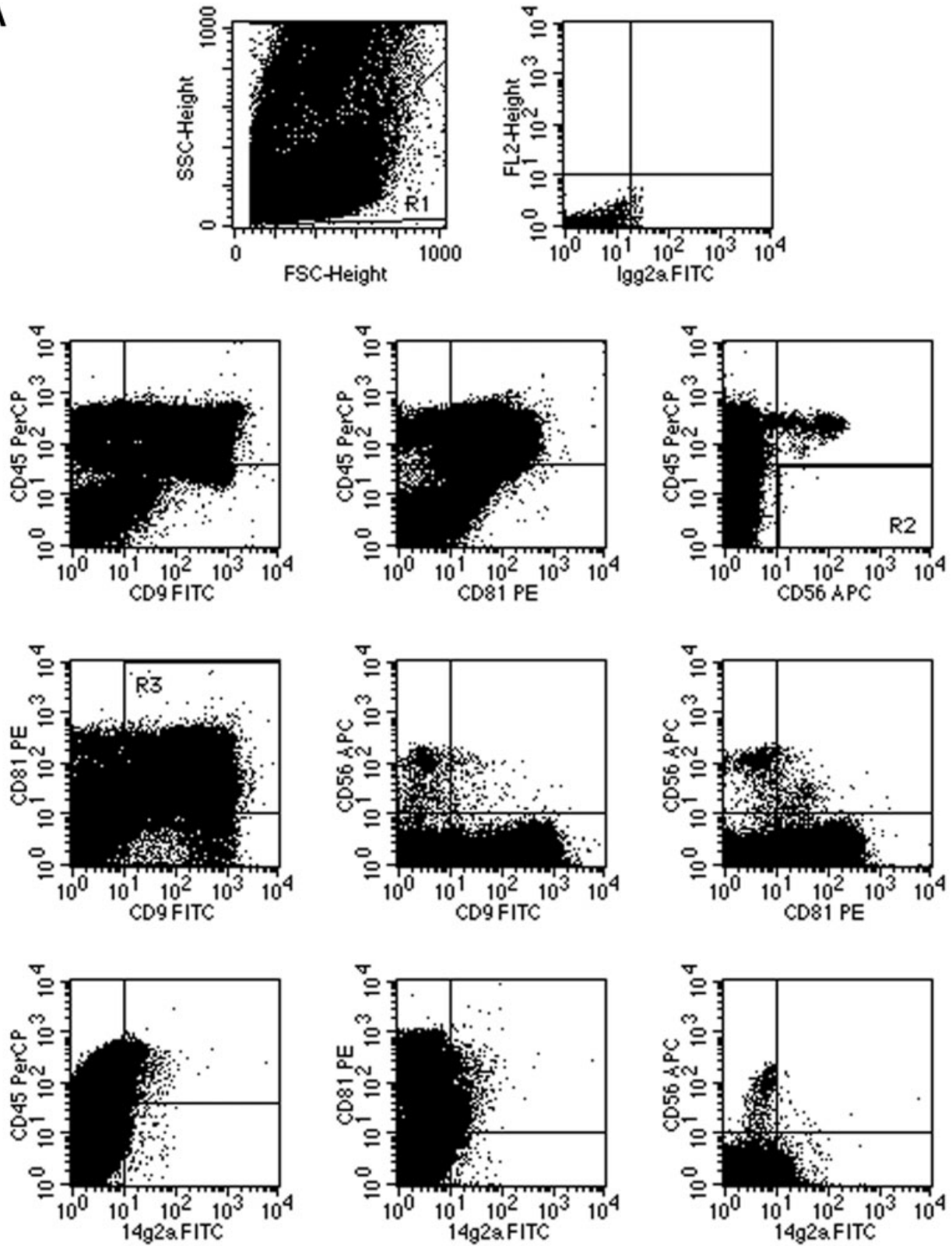
A

FIG. 3. Example of a bone marrow in which the residual neuroblastoma cells were found only after immunocytochemical analysis. **A:** Flow cytometric results. No tumor cells were detected. A total of 5×10^5 cells/tube were analyzed. **B:** Immunocytochemical results. One single cell and a cluster of three tumor cells fulfilling the criteria were found.

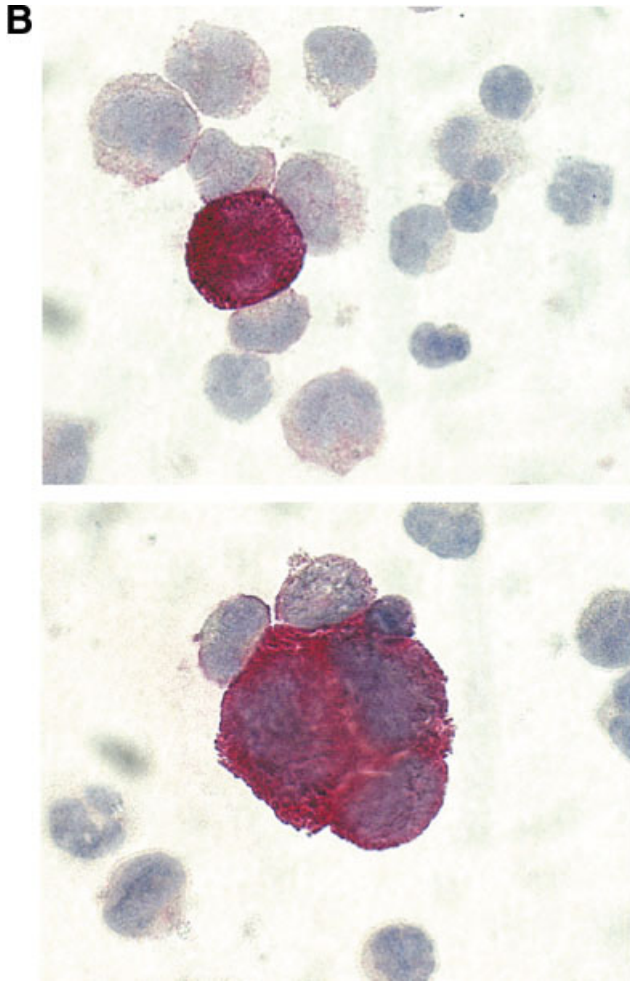


FIG. 3. (continued)

from the DIG tumor, containing mature gangliocytes, were $CD9^+/CD81^+/CD45^-/CD56^+$ and $GD2^+/CD81^+/CD45^-/CD56^+$. Because the CD56 expression on the DIG cells was as high as the CD56 expression on NB cells, it was impossible to differentiate between them. The GD2 expression on the ganglioneuroma cells was very weak. Others have reported the absence of GD2 in more differentiated ganglioneuroblastomas and ganglioneuromas (18,20).

In six samples, discordant flow cytometric and immunocytochemical results were obtained. In these samples, taken during therapy, only a few residual NB cells were found after immunocytochemical analysis. The discrepancy was probably due to sample variability and the difference in the number of analyzed cells. Because the conventional immunocytochemical assay was considered the gold standard for minimal residual disease detection in patients with an undifferentiated NB, priority was given to this test and 3×10^6 cells were immunocytochemically evaluated. The remaining cells were analyzed with the two four-color flow cytometric assays. Because BM sam-

ples taken during therapy are often aplastic, in some cases only 2×10^4 cells could be flow cytometrically analyzed. This means a sensitivity of 0.1% was reached. Seeger et al. reported that a sensitivity of 0.1% (100 NB cells/ 10^5 BM cells) after three to four cycles of therapy most likely represents the clinically useful threshold for patients (4). Children who demonstrated less sensitivity survived, whereas those with greater sensitivity died. Therefore, the collection of 1×10^4 cells/tube was considered adequate.

Two combinations of different monoclonal antibodies are currently used to screen for residual NB cells. This means that at least 2×1.10^6 cells must be analyzed to reach a sensitivity of 0.001%. This amount of cells is sometimes not available. In the near future, we plan to develop a five-color flow cytometric assay so that we can decrease the amount of required cells by one-third.

The discrepancy between the immunocytochemical and flow cytometric results can also be explained by a difference in sensitivity. Our immunocytochemical test has a targeted sensitivity of 0.0001%. This is approximately 10 to 100 times more sensitive than that reported in previous publications (4,28,36,37). The sensitivity of the assay is not limited by the technique but by the number of analyzed cells. When enough cells are analyzed, a high sensitivity can be reached. The Poisson distribution, $f(X) = \mu^X \times e^{-\mu}/X!$, can be used to calculate the statistics of tumor detection (28). At least 3×10^6 cells must be analyzed to have a 95% chance to detect one tumor cell in a million normal mononuclear cells. In previous reports, fewer than 3×10^6 cells were analyzed, which decreased the sensitivity of the assays. In contrast, the flow cytometric assay has a sensitivity of 0.01% to 0.001% based on spiking experiments. This corresponds with previously published reports (12,14,18). In our study, a sensitivity of 0.1% to 0.2% was reached because at least 2×10^4 cells were flow cytometrically analyzed.

In conclusion, the sensitivity of the flow cytometric assays is lower than that of the immunocytochemical assay in spiking experiments. However, the flow cytometric assays can be used to screen for residual cells in clinical samples with a sensitivity of one NB cell in 10^4 to 10^5 normal mononuclear cells. They are less labor intensive and time consuming than the immunocytochemical test. The results are available only a few hours after collection, and the technique is simple and cost effective. Further, in contrast to our immunocytochemical assay, the four-color flow cytometric tests can be used to screen for residual NB cells in case of a GD2-negative primary tumor. Therefore, we recommend flow cytometry for the detection of residual NB cells in BM.

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